Characterization of avian influenza viruses. Designation of a newly recognized haemagglutinin

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Studies with specific antisera to the haemagglutinin and neuraminidase antigens of all the influenza A subtypes show that A/turkey/Wisconsin/66 influenza virus, originally included in the Hav6 subtype, does not react in either haemagglutinin inhibition or immunodiffusion tests with antisera to Hav6. It is therefore proposed that A/turkey/Wisconsin/66 be placed in a new subtype designated Hav9. The neuraminidase antigens of the Hav6 subtype were further characterized and were shown to be N1, N2, Neq2, and Nav5 subtypes. Hav6 influenza viruses isolated from turkeys over an 11-year period showed little antigenic drift. The haemagglutinin and neuraminidase of A/shearwater/Australia/72 (Hav6Nav5) were identical with those of a virus isolated 8 years previously from a turkey in California: A/turkey/California/64 (Hav6Nav5).

According to the revised system of nomenclature for influenza viruses (*1*) the avian viruses, which form the largest group of animal influenza viruses, have been divided hitherto into 8 haemagglutinin subtypes (Hav1–Hav8). Five neuraminidase subtypes were originally described and a sixth has recently been added (Nav1–Nav6) (*18*). In this system of nomenclature, A/turkey/Wisconsin/66 was included in the Hav6 subtype (*6, 9, 10, 11, 12*). However, with the development of specific antisera to the isolated haemagglutinin and neuraminidase antigens, it has become evident that the Hav6 virus subtype is not homogeneous, since some viruses originally included in this subtype do not react with the antisera to the haemagglutinin of Hav6 subtype.

In the present communication we present evidence indicating that A/turkey/Wis/66 does not belong to the Hav6 subtype of avian influenza viruses and should be placed in a new subtype designated Hav9. In addition, the neuraminidase antigens of two strains of the new subtype and of a number of strains of the Hav6 subtype—previously not completely determined—were characterized with monospecific antisera.

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MATERIALS AND METHODS

Viruses

The following strains of influenza A viruses were used: turkey/Canada/63 (Wilmot strain); turkey/California/64; turkey/Massachusetts/3740/65; turkey/England/66; turkey/Washington/67; duck/Germany/1868/68; duck/Pennsylvania/486/69; shearwater/Australia/72; turkey/Wisconsin/66; turkey/California/66; turkey/Ontario/5614/64; turkey/Ontario/5050/65; turkey/Ontario/5379/66; turkey/Ontario/4689/67; turkey/Ontario/4054/68; and turkey/Ontario/8009/74.

The viruses were grown in the allantoic sac of 11-day-old chick embryos. Virus was purified from allantoic fluid by adsorption to and elution from chicken erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10–40% sucrose in 0.15 mol/litre NaCl) as previously described (*7*). Recombinant influenza viruses (antigenic hybrids) were prepared as previously described (*14*).

Antisera

Antisera to the isolated haemagglutinin and neuraminidase antigens were prepared in goats (*17*).

Table 1. Interrelationships of the Hav6 influenza viruses, shown by the haemagglutination inhibition test[a]

Sera	HI titres to the influenza viruses: T/Can 63	T/Eng 66	T/Wash 67	T/Cal 64	T/Mass 65	D/Ger 68	D/Pen 69	Sh/Aus 72	T/Wis 66	T/Wis/66 Eq1	T/Cal 66
A/turkey/Canada/63 (Wilmot)	590	40	40	40	<	<	<	<	<	<	<
A/turkey/England/66	64	450	450	360	70	260	360	200	<	<	<
A/turkey/Washington/67	70	450	240	440	180	190	250	250	<	<	<
A/turkey/California/64	40	440	64	550	180	450	450	440	<	<	<
A/turkey/Massachusetts/65	<	60	64	128	440	80	40	80	<	<	<
A/duck/Germany/1868/68	<	250	128	150	160	720	190	250	<	<	<
A/duck/Pennsylvania/69	<	50	56	64	<	40	64	<	<	<	<
A/duck/Pennsylvania/69(H) [b]	1100	750	1500	1200	250	200	1600	300	<	<	<
A/turkey/Massachusetts/65 (H) [b]	560	1000	2500	1800	3000	320	2600	300	<	960	3500
A/turkey/Wisconsin/66	<	<	<	<	<	<	<	<	1800	2500	2000
A/turkey/Wisconsin/66 (H)-Eq1 (N)	<	<	<	<	<	<	<	<	2000	3500	2300
A/turkey/Wisconsin/66 (H)-Sw/30 (N)	<	<	<	<	<	<	<	<	3800		

[a] The figures represent the reciprocal of the dilution inhibiting 3 out of 4 agglutinating doses of virus; < = less than 20.
[b] Specific sera to the isolated haemagglutinin.

Rat and rabbit hyperimmune sera to intact influenza viruses were prepared as previously described (*4*, *13*, *15*).

Serological tests

Haemagglutinin (HA) titrations and haemagglutination inhibition (HI) tests were done as previously described (*5*). In HI tests the dilutions of antiserum were allowed to interact with antigen for 60 min at 20°C before the addition of chicken erythrocytes. Neuraminidase (NA) assays and neuraminidase inhibition (NI) tests were carried out as recommended by the World Health Organization (*2*). Immunodiffusion tests were performed in 1.5% agarose A37 in phosphate-buffered saline containing 0.1% sodium lauroylsarcosinate NL97 and 0.08% sodium azide. The purified virus (HA >6.0 $\log_{10}$ units/ml was disrupted with sodium lauroylsarcosinate NL97 and the precipitin lines were photographed without staining. Sodium lauroylsarcosinate (0.1%) was also added to the antisera to prevent nonspecific zones of precipitation.

RESULTS

Cross-reactions mediated through the haemagglutinin

Haemagglutination inhibition tests with antisera to intact viruses, with recombinants containing an irrelevant neuraminidase, or with " monospecific " antisera to isolated haemagglutinin, showed that A/turkey/Wis/66 and A/turkey/Cal/66 gave no reactions with a number of viruses that belong to the Hav6 subtype (Table 1, Fig. 1). The remaining influenza viruses examined were related to each other immunologically and formed a relatively homogeneous group except for A/turkey/Can/63, which was somewhat different from the other members of the group. However, with specific sera to the isolated haemagglutinin of the reference strain (A/turkey/Mass/65), all the viruses except A/turkey/Wis/66 and A/turkey/Cal/66 differed from each other by a factor of less than five.

Specific antisera to the haemagglutinin of A/duck/Pen/69 gave two lines of precipitation with A/turkey/Mas/65 and A/duck/Pen/69 viruses (Fig. 1); this reaction has been observed before with specific antisera (*8*) and may be related to the different antigenic determinants on the haemagglutinin antigen. The faint line of precipitation observed between antisera to A/turkey/Mas/65 and A/turkey/Wis/66 is probably due to host antigen covalently attached to the haemagglutinin antigens. A/turkey/Wis/66 and A/tur-

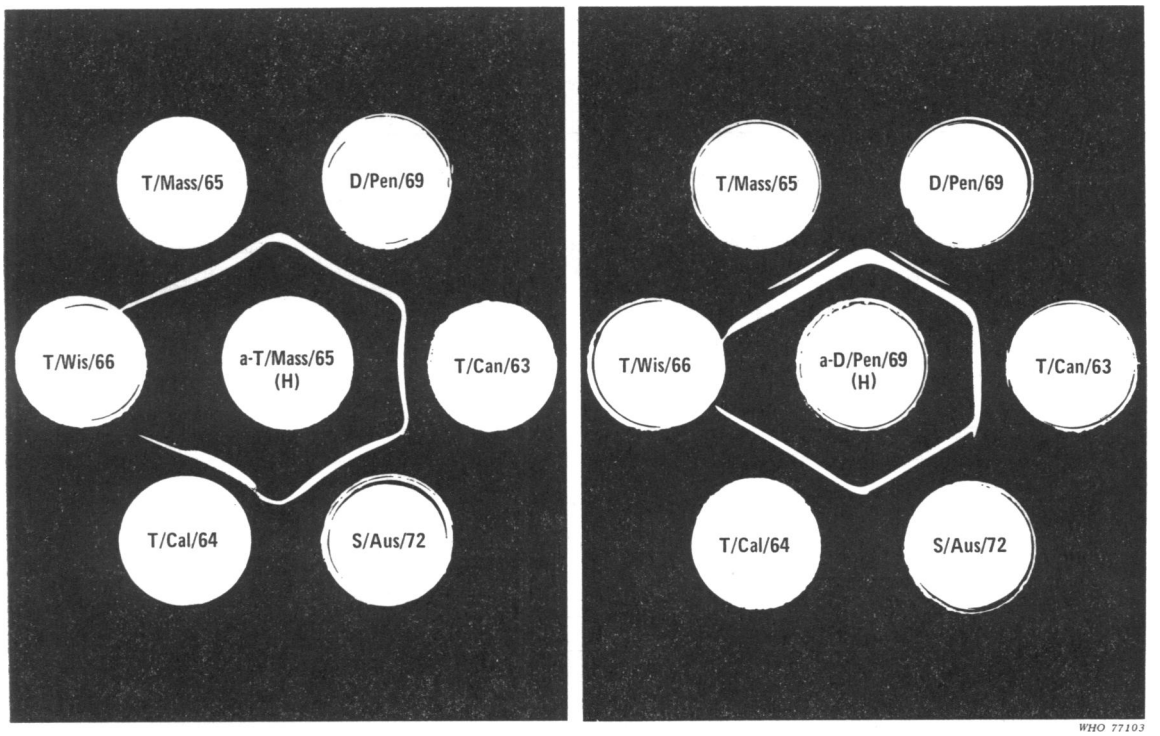

Fig. 1. Double immunodiffusion showing that the haemagglutinin antigens of A/turkey/Mass/66 and A/turkey/Wis/66 are not related.

Left centre: antiserum to the isolated haemagglutinin of A/turkey/Mass/65.
Right centre: antiserum to the isolated haemagglutinin of A/duck/Pen/69.
Outer: Influenza viruses as indicated, disrupted with sodium lauroylsarcosinate.

key/Cal/66 did not react with any of the known human, animal, or avian influenza virus haemagglutinin antigens when tested with specific antisera in HI tests (results not shown).

Hav6 influenza viruses isolated over an 11-year period from turkeys in Ontario, Canada, were examined for evidence of antigenic drift (Table 2). By means of specific antisera to the haemagglutinin of A/turkey/Mass/65 and A/duck/Pen/69 it can be seen that A/turkey/Can/63 differs from some of the later isolates by a factor of 5–10, but that the remaining viruses show no significant antigenic drift.

Characterization of the neuraminidase antigens

The neuraminidase antigens of the avian influenza viruses were characterized with specific sera to the neuraminidase of all known human, animal, and avian strains. Table 3 shows that the viruses in the Hav6 subtype have N1, N2, Nav5, or Neq2 neuraminidase antigens, while A/turkey/Wis/66 and A/turkey/Cal/66 both have N2 neuraminidase. Thus the human type neuraminidase (N1 and N2), as well as an equine (Neq2) and an avian (Nav5) neuraminidase, are found in this group of influenza viruses. A virus isolated from turkey (A/turkey/Cal/64) was shown to possess Nav5 neuraminidase (Table 3, Fig. 2).

DISCUSSION

The above-mentioned studies show that A/turkey/Wis/66 influenza virus, originally included in the Hav6 subtype, does not react in either HI or gel

Table 2. Antigenic drift in Hav6 influenza viruses [a]

Viruses	HI titres with antisera to the isolated HA of:	
	A/turkey/Mass/65 a-Hav6	A/duck/Pen/69 a-Hav6
A/turkey/Canada/63 (Wilmot) [Hav6Neq2]	200	800
A/turkey/Ontario/5614/64 [Hav6 N2]	2000	640
A/turkey/Ontario/5050/65 [Hav6 N2]	2000	3500
A/turkey/Ontario/5379/66 [Hav6 N2]	5000	2500
A/turkey/Ontario/4689/67 [Hav6 N1]	700	1300
A/turkey/Ontario/4054/68 [Hav6 N2]	1000	1200
A/turkey/Ontario/8009/74 [Hav6 N1]	1800	1800

[a] The figures represent the reciprocal of the dilution inhibiting 3 out of 4 agglutinating doses of virus.

Table 3. Interrelationships of the neuraminidases of a group of avian influenza viruses

Influenza viruses	Antisera to the influenza virus neuraminidases [a]				Designation
	N1	N2	Neq2	Nav5	
A/duck/Germany/1868/68	+	—	—	—	Hav6N1
A/duck/Pennsylvania/486/69	+	—	—	—	Hav6N1
A/turkey/England/66	—	+	—	—	Hav6N2
A/turkey/Washington/67	—	+	—	—	Hav6N2
A/turkey/Massachusetts/65	—	+	—	—	Hav6N2
A/turkey/California/64	—	—	—	+	Hav6Nav5
A/shearwater/Australia/72	—	—	—	+	Hav6Nav5
A/turkey/Canada/63	—	—	+	—	Hav6Neq2
A/turkey/Wisconsin/66	—	+	—	—	Hav9N2
A/turkey/California/66	—	+	—	—	Hav9N2

[a] Antisera to the isolated neuraminidase of all the designated subtypes were tested, but only those showing inhibition are presented.
+ = inhibition of neuraminidase activity.
— = no inhibition.

diffusion tests with specific antisera to the haemagglutinin of the Hav6 subtype. Since this virus did not react with any of the known human, animal, or avian influenza subtypes, it is proposed that it should be placed in a new subtype designated Hav9. It is not surprising that A/turkey/Wis/66 was originally included in the Hav6 subtype (*6*, *10*, *11*, *12*), since both A/turkey/Mas/65 and A/turkey/Wis/66 possess N2 neuraminidase, and antibodies to the common antigen would cause steric inhibition in HI tests.

Avian influenza viruses of the Hav6 subtype isolated from turkeys in Ontario, Canada, over an 11-year period showed little antigenic drift. The neuraminidase of A/turkey/Cal/64 (Hav6Nav5) is identical with that of a virus isolated from migrating pelagic birds in Australia in 1972 (A/shearwater/Australia/72 (Hav6Nav5) (*3*). This observation suggests that migratory birds may be involved in the dissemination of influenza viruses over vast areas. Most of the avian influenza viruses studied possessed

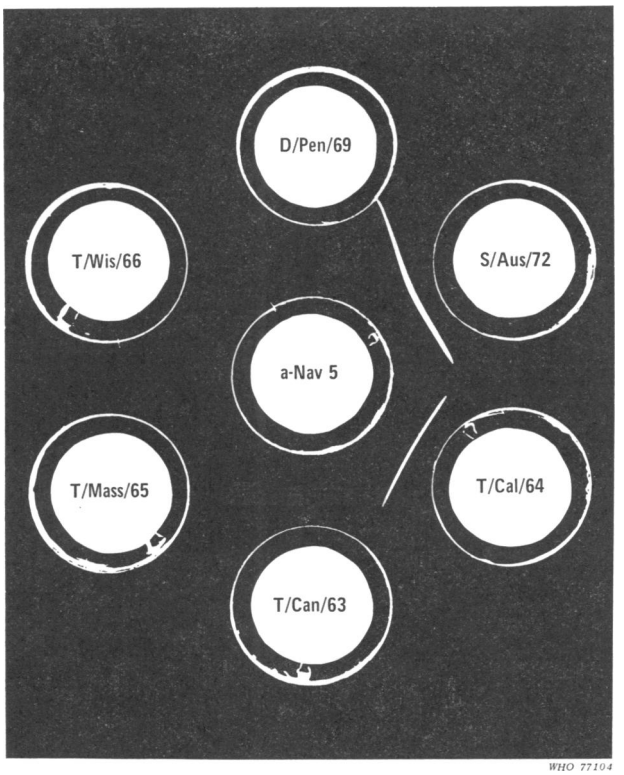

Fig. 2. Double immunodiffusion showing that A/shearwater/Aus/72 and A/turkey/Cal/64 possess Nav5 neuraminidase.

Centre: antiserum to the isolated neuraminidase of A/shearwater/Aus/72.

Outer: influenza viruses as indicated, disrupted with sodium lauroylsarcosinate.

N1 or N2 neuraminidase antigens closely related to the antigens of influenza viruses isolated from man. It seems unlikely, at this time, that migrating birds are involved in the spread of human influenza viruses, but they are a potential source of genetic information from which " new " human influenza viruses could arise by recombination (*16*).

Evidence of the role of influenza viruses from animals in the origin of human strains remains circumstantial. It is still not known whether the number of influenza virus subtypes in the world is limited or not. The characterization of all influenza viruses isolated from animals will eventually answer this question and may indicate whether it is possible to isolate a future human pandemic strain before the virus appears in man.

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RÉSUMÉ

CARACTÉRISATION DES VIRUS GRIPPAUX AVIAIRES DÉSIGNATION D'UNE NOUVELLE HÉMAGGLUTININE

Des études effectuées avec des immunsérums spécifiques contre les antigènes, hémagglutinines et neuraminidases, de tous les sous-types de virus grippaux A montrent que le virus grippal A/turkey/Wisconsin/66, qui avait été initialement rangé dans le sous-type Hav6, ne réagit pas avec les sérums anti-Hav6 dans les réactions d'inhibition de l'hémagglutination, ni dans celles d'immunodiffusion. Il est donc proposé de placer A/turkey/Wisconsin/66 dans un nouveau sous-type désigné comme Hav9. Les caractéristiques des antigènes neuraminidasiques du sous-type Hav6 ont été étudiées plus à fond; il a été montré qu'il s'agissait des sous-types N1, N2, Neq2, et Nav5. Il est apparu que les virus grippaux Hav6 isolés des dindons sur une période de plus de 11 années ne présentaient qu'un glissement antigénique réduit. L'hémagglutinine et la neuraminidase de A/shearwater/Australia/72 (Hav6Nav5) étaient identiques à celles d'un virus isolé 8 ans auparavant d'un dindon en Californie: A/turkey/California/64 (Hav6Nav5).

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